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Role of nucleus accumbens core but not shell in incubation of methamphetamine craving after voluntary abstinence

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Abstract - (243**)**

We recently introduced an animal model to study incubation of drug craving after prolonged voluntary abstinence, mimicking the human condition of relapse after successful contingency management treatment. Here we studied the role of the nucleus accumbens (NAc) in this model.

We trained rats to self-administer a palatable solution (sucrose+maltodextrin 1%, 6 h/day, 6 days) and methamphetamine (6 h/day, 12 days). We then evaluated relapse to methamphetamine seeking after 1 and 15 days of voluntary abstinence, achieved via a discrete choice procedure between the palatable solution and methamphetamine (14 days). We used RNAscope in-situ hybridization to quantify the co-labeling of the neuronal activity marker Fos, and dopamine Drd1- and Drd2-expressing medium spiny neurons (MSNs) in NAc core and shell during the incubation tests. Next, we determined the effect of pharmacological inactivation of NAc core and shell by either $GABA_A$ and $GABA_B$ agonists (muscimol+baclofen, 50+50 ng/side), Drd1-Drd2 antagonist (flupenthixol, 10 µg/side) or the selective Drd1 or Drd2 antagonists (SCH39166 1.0 µg/side or raclopride 1.0 µg/side) during the relapse tests.

Incubated methamphetamine seeking after voluntary abstinence was associated with a selective increase of Fos expression in the NAc core, but not shell, and Fos was co-labeled with both Drd1- and Drd2-MSNs. NAc core, but not shell, injections of muscimol+baclofen, flupenthixol, SCH39166, and raclopride reduced methamphetamine seeking after 15 days of abstinence.

Together, our results suggest that dopamine transmission through Drd1 and Drd2 in NAc core is critical to the incubation of methamphetamine craving after voluntary abstinence.

Key words: methamphetamine, self-administration, contingency management, relapse, palatable food, nucleus accumbens, dopamine receptor, Fos

Introduction (628 words)

Despite decades of research on the neurobiological mechanisms of psychostimulant addiction, the only effective treatments are based on behavioral intervention strategies which harnesses operant principles to promote voluntary abstinence [1,2]. One of these methods is contingency management in which drug abstinence is reinforced and maintained via delivery of alternative incentives (e.g., monetary vouchers) [3]. However, when contingency management is discontinued most addicts relapse to drug use [4,5].

At present, the brain mechanisms underlying relapse after cessation of contingency management are unknown. Based on the seminal studies of Lenoir et al., [6] and Ahmed et al., [7], showing that most rats prefer palatable food over cocaine in a discrete choice setting, we recently introduced a choice-based rat model of relapse after voluntary abstinence [8]. In this procedure, we first trained food-sated rats to self-administer palatable food (the alternative nondrug reward) and then methamphetamine for several weeks. We then assessed relapse to drug seeking at an early and late abstinence day. Between methamphetamine self-administration and the test on late abstinence day, rats were exposed to daily mutually exclusive choice sessions between the drug and palatable food [9]. Under these 'contingency management' conditions, like human addicts, male and female rats choose to abstain from methamphetamine or heroin [8,10,11]. However, after discontinuing the palatable food, the rats' drug seeking response progressively increased during abstinence. This phenomenon, termed 'incubation of drug craving', was first observed after home cage forced abstinence conditions in rats trained to self-administer cocaine [12], and subsequently with other abused drugs [13,14]. Evidences of this phenomenon are also available in humans for nicotine [15], methamphetamine [16], alcohol [17,18] and cocaine [19].

In our initial mechanistic characterization of the choice-based rat model of relapse, we identified: 1) a critical role of dopamine receptor 1-expressing (Drd1) neurons in the CeA and the glutamatergic projection from anterior insular cortex to central amygdala [20] in relapse to methamphetamine seeking after voluntary abstinence; 2) a critical role of dorsomedial striatum neuronal ensembles, in incubation of methamphetamine craving after voluntary abstinence [10]. In the current study, we assessed the role of nucleus accumbens (NAc) subregions (core and shell) in incubation of

methamphetamine craving after food choice-induced voluntary abstinence. Anatomically, the NAc core and shell are distinguished on the basis of the morphology and projections although both subregions are primarily constituted of two neuronal populations: dopamine receptors Drd1- and Drd2-expressing medium spiny neurons (MSNs) that further differ in the neuropeptide expression profile [21].

We studied the NAc core because previous studies showed that incubated cocaine craving after forced abstinence is associated with an altered firing in this area [22,23]. Furthermore, incubated cocaine and methamphetamine craving after forced abstinence is reduced by inhibiting calcium-permeable AMPA receptor (CP-AMPARs) transmission in NAc core [24-26] or, for cocaine, via weakening the prelimbic cortex to NAc core pathway [27].

We studied the role of the NAc shell because**,** similarly to the core subregion, increased CP-AMPARs transmission is also evident after prolonged abstinence from cocaine self-administration [27-30]. Furthermore, incubated cocaine craving is reduced by selectively weakening the basolateral amygdala to NAc shell pathway [31], or simultaneous weakening of the infralimbic to Drd1-MSNs shell and ventral hippocampus to Drd1-MSNs shell pathways [32].

In the current study, we first used an RNAscope in situ hybridization (ISH) method used in our previous studies [10,20,33] to determine whether incubation of methamphetamine craving is associated with activation of Drd1 and Drd2 in NAc core and shell, assessed by the activity marker Fos [34]. Next, we used site-specific reversible inactivation with $GABA_A$ and $GABA_B$ agonists (muscimol + baclofen [35]), Drd1-Drd2 antagonist (flupenthixol [36]) and selective Drd1 and Drd2 receptor blockade with SCH39166 [37] and raclopride [38] to determine the causal role of these receptors in NAc core and shell in incubation of methamphetamine craving after food choice-induced voluntary abstinence.

Materials and Methods

Subjects, Surgery (intravenous and intracranial), Drugs, Intracranial injections, RNAscope in situ hybridization assay, Self-administration apparatus, Procedures, Palatable solution self-administration, Methamphetamine self-administration, Discrete choice procedure, Voluntary abstinence and Relapse test: see Supplemental Online Material.

Our procedures followed the guidelines of the national law (DL 26/2014) on the use of animals for research based on the European Communities Council Directive (2010/63/UE), and were approved by the ethics committee of the Italian Ministry of Health (license/approval ID #: 705/2018-PR) and by the local Ethical Committee of the Santa Lucia Foundation.

Specific experiments

Experiment 1: Fos expression in NAc core and shell during relapse tests after voluntary abstinence

In experiment 1 (Exp. 1), we determined whether incubation of methamphetamine craving is associated with increased neuronal activity, as assessed by the activity marker Fos [39] in NAc core and shell. We also determined the cell type of the Fos-positive neurons in the two subregions by co-labeling Fos with Drd1 and Drd2 [10,20,33,40]. We used four groups of rats (n=3–5/group) in an experimental design that included the between-subject factors of Test condition (no-test, relapse test) and Abstinence day (days 1 and 15). The experiment consisted of three phases: training, discrete choice voluntary abstinence, and relapse tests.

Training. We first trained rats to self-administer first the palatable solution (sucrose 1% + maltodextrin 1%; SM 1%, 0.4 ml/reward delivery; 6 h/d, 6 d) and then methamphetamine (6 h/d, 12 d; 0.1 mg/kg/infusion; 0.1 ml/infusion). The SM1% solution delivery and the methamphetamine infusions were paired with the presentation of 20-second discrete light cues (triple-light or white-light, respectively).

Discrete choice procedure. We determined SM1% versus methamphetamine choice after every three consecutive drug self-administration sessions in all groups (three choice tests, during the training phase) and for 14 days (voluntary abstinence) preceding the abstinence day 15 relapse test.

Relapse tests. We tested the rats for methamphetamine seeking under extinction conditions on abstinence days 1 or 15. The relapse tests were performed under extinction conditions in the presence of the house light. Active lever presses during testing, the operational measure of drug seeking in incubation of drug craving studies [13,14] resulted in contingent presentations of the white-light cue previously paired with methamphetamine, but not methamphetamine delivery. Immediately after the 60-minute relapse tests, we anesthetized the rats and processed their brains for the RNAscope in situ hybridization assay. For the no-test rats, we brought them to the perfusion room

from their home cage and perfused them on the same day and time as the relapse test rats. We matched the rats in the different groups for methamphetamine intake during the training phase.

Exp. 2: Effect of the NAc core or shell muscimol + baclofen (M+B) inactivation on incubation of methamphetamine craving after voluntary abstinence

We performed intravenous surgeries on the rats and implanted them with bilateral guide cannulas 1 mm above the NAc core or shell (see SOM). The experimental procedure is identical to the one reported in Exp. 1, with the exception that we microinjected M+B in NAc before the relapse tests. We also habituated the rats to the injection procedure for 3 days during the discrete choice procedure. We used eight groups of rats (n=6–8/group) in an experimental design that included the between-subject factors of Brain region (core, shell), Abstinence day (1, 15) and Muscimol + Baclofen dose (M+B; 0, 50 ng+50 ng/ 0.5 µl/side).

Relapse tests. We determined the effect of reversible inactivation of the NAc core and shell on extinction responding on abstinence days 1 or 15. We injected bilaterally either vehicle (saline) or M+B (50 ng+50 ng/0.5 µl/side) into NAc core or shell 15 minutes before the 60-minute extinction test. Finally, to ensure that the effect of core inactivation by M+B on extinction responding during the late withdrawal test was not due to motor deficits, we re-trained 12 rats from the NAc core group to self-administer the SM1% solution after they completed core injections and extinction tests on abstinence day 15. We re-trained them for 6 days (6 h/d) for the SM1% solution and then injected them vehicle or M+B (50 ng+50 ng/0.5 µl/side) into NAc core, 15 minutes before the 60-minute self-administration session.

Exp. 3: Effect of NAc core SCH39166 or raclopride injections on incubation of methamphetamine craving

In Exp. 1, we found selective time-dependent increases in Fos expression in NAc core, but not in shell, and that Fos was co-labeled with both Drd1 and Drd2. Moreover, in Exp. 2 we found a selective role of NAc core, but not shell. Based on these results, in Exp. 3 we determined the causal role of NAc core's dopamine receptors in the incubation of methamphetamine craving after food choice-induced voluntary abstinence. We used three groups of rats (n=6–8/group) in an experimental design that included the between-subject factors of Drug condition (vehicle, SCH39166, raclopride). We

excluded n=2 due to cannula's misplacement. The experiment procedure is identical to the one reported in Exp. 2 (see SOM).

Relapse tests. We tested rats for methamphetamine seeking under extinction conditions on abstinence day 15. We injected either the Drd1 antagonist SCH39166 (1.0 µg/0.5 µl/side) or the Drd2 antagonist raclopride (1.0 µg/0.5 µl/side) into the core 15-min before testing. The length of the test session was 60 minutes. We matched the rats in the different groups for methamphetamine intake during the training phase. Finally, to verify that SCH39166 and raclopride, at the dose used in the relapse tests, do not non-selectively decrease operant responding, we re-trained 21 rats after the day 15 testing to lever press for the SM1% for 6 h/d. After 6 days of training sessions, we injected the rats with vehicle (0.5 µl/side), SCH39166 (1.0 µg/0.5 µl/side) or raclopride (1.0 µg/0.5 µl/side) 10 minutes before the 60-minute test session.

Exp. 4: Effect of NAc core flupenthixol injections on incubation of methamphetamine craving

In Exp. 3, we found that a reversible pharmacological inactivation of Drd1 or Drd2 MSNs in the NAc core decreased methamphetamine seeking during late (day 15) abstinence. In both cases we observed about 60% reduction of drug-seeking responding. Based on these results, in Exp. 4 we determined whether by inactivating both Drd1 and Drd2 MSNs with the Drd1-Drd2 antagonist cis-(Z)-Flupenthixol dihydrochloride (flupenthixol) will cause total suppression of methamphetamine seeking on day 15. In a separate group of rats we first determined the dose of flupenthixol, injected in the NAc core, that did not affect SM1% solution-reinforced responding, (see SOM).

We used two groups of rats (n=6–8/group) in an experimental design that included the between-subject factors of dopamine antagonist condition (vehicle, flupenthixol). We excluded two rats due to cannula's misplacement. The experiment procedure is identical to the one reported in Exp. 2 (see SOM).

Relapse tests. We tested rats for methamphetamine seeking under extinction conditions on abstinence day 15. We injected either the vehicle or flupenthixol (10 µg/0.5 µl/side) into the core 15-min before testing. The length of the test session was 60 minutes. We matched the rats in the different groups for methamphetamine intake during the training phase.

Statistical analysis

Behavioral data. We analyzed the data with the statistical program SPSS Statistics using GLM module (IBM, version 23). For the self-administration training, we analyzed the amount of SM1% solution rewards and methamphetamine infusions separately with a repeated-measures ANOVA, using the within-subjects factor of Session. For the choice sessions during the training phase and the food choice-induced voluntary abstinence we analyzed the data with a repeated-measures ANOVA, using the within-subjects factors of Reward Type (SM1% solution or methamphetamine) and Choice Session.

For the relapse test in Exp. 1 we analyzed active lever pressing using one-way ANCOVA with the between-subjects factor of Abstinence Day (1 or 15), and we included the inactive lever presses as covariate. For the relapse test in Exp. 2, we analyzed data using the between-subjects factors of Abstinence day (1 or 15) and M+B dose (0 or 50+50 ng). For the relapse tests in experiment 3-4, we used one-way ANCOVA with the between-subjects factors of dopamine antagonist condition (Exp. 3: vehicle, SCH39166, raclopride; Exp. 4: vehicle, flupenthixol) and the inactive lever presses were included as a covariate. For Exp. 2, 3 and 4, we also used a mixed ANOVA to analyze the 60-minute time course of active lever responding for day 15, using the between-subjects factor of Drug condition and the within-subjects factor of Session minutes (20, 40, 60). For the dose effect curve of flupenthixol, we analyzed the data using a repeated-measures ANOVA with the within-subject factor of flupenthixol Dose (0, 10, 20, 30 and 40 µg) and the Least Significant-Difference test for multiple comparisons.

RNAscope and immunohistochemistry data. In Exp. 1 we analyzed the data with the between-subjects factors of Abstinence Day (1 or 15) and Test Condition (test or no-test).

In the Supplementary Online Material (SOM) we offer a summary of the statistical analysis. In the figures we only report significant effects that are critical for data interpretation. We followed up on significant main and interaction effects ($p<0.05$) using Fisher PLSD post-hoc tests.

Results

Exp. 1: Fos expression in NAc core and shell during relapse tests after voluntary abstinence

The timeline of Exp. 1 is shown in Fig. 1A.

SM1% and methamphetamine training: The rats increased the rewards intake over sessions (Fig.1B) and extended daily access (6 hours/day) to methamphetamine led to escalation of drug intake [8,10]. The statistical analysis showed a significant effect of session for SM1% solution ($F_{5,75}$=21.903, p<0.001) and methamphetamine ($F_{11,165}$=20.5, p<0.001). During the three discrete choice sessions, the rats showed a strong preference for the SM1% solution ($F_{1,15}$=164.2, p<0.001; Fig. 1C).

Voluntary abstinence: During the 14 days of food choice-induced abstinence the rats showed a strong preference for the SM1% solution, with an almost complete suppression of the methamphetamine self-administration during the voluntary abstinence (Fig. 1D). The statistical analysis showed a significant effect of reward type ($F_{1,7}$=259.1, p<0.001).

Relapse test: Methamphetamine seeking in the extinction tests was higher at abstinence day 15 than after 1 abstinence day, demonstrating incubation of methamphetamine craving after voluntary abstinence (Fig. 1E). The ANCOVA of active lever presses (inactive lever as covariate) showed a significant effect of Abstinence Day ($F_{2,7}$=87.4, p<0.001).

Fos and Fos + Drd1 or Drd2 RNAscope double-label data: Representative pictures of Fos/Drd1/Drd2 triple labeling by RNAscope in situ hybridization are shown in Fig. 1F. Fos expression in the relapse test was higher at abstinence day 15 relative to abstinence day 1 in NAc core but not in NAc shell. (Fig. 1G). The statistical analysis, which included the between-subjects factors of Abstinence Day and Test condition showed a main effect of both factors and the interaction between them for NAc core [Abstinence Day ($F_{1,12}$=21.1, p=0.001); Test ($F_{1,12}$=25.2, p<0.001); Abstinence Day X Test interaction ($F_{1,12}$=13.5, p=0.003)]. We found no evidence for cell-type specificity of the activated (Fos-positive) NAc core neurons on abstinence day 15 (Fig. 1H, 1I upper and lower panels; for statistics refer to Supplementary Table 1).

Exp. 2: Effect of the NAc core or shell muscimol + baclofen (M+B) inactivation on incubation of methamphetamine craving

The timeline of the experiment is shown in Fig. 2A.

SM1% solution and methamphetamine training: The rats increased the rewards intake over sessions (Fig. 2B). The statistical analysis showed a significant effect of session for SM1% solution

($F_{11,250}$=52.6, p<0.001) and methamphetamine ($F_{11,550}$=81.2, p<0.001). During the three discrete choice sessions the rats showed a strong preference for the SM1% solution over methamphetamine that increased over time [Session ($F_{2,100}$=6.6, p=0.002): Reward ($F_{1,50}$=76.5, p<0.001); Reward X Session ($F_{2,100}$=9.3, p<0.001; Fig. 2C)]. We did not observe differences between groups.

Voluntary abstinence: During the 14 days of food choice-induced voluntary abstinence the rats showed a strong preference for the SM1% solution, with an almost complete suppression of methamphetamine self-administration during the voluntary abstinence that increased over time (Fig. 2D). The statistical analysis showed a significant effect of Reward type ($F_{1,26}$=596.7, p<0.001) and Session ($F_{13,338}$=3.7, p<0.001), and the interaction Reward X Session ($F_{13,338}$=2.3, p=0.006).

Relapse test: NAc core injections of M+B decreased methamphetamine seeking in the extinction tests on abstinence day 15 but not abstinence day 1 (Fig. 2E). The statistical analysis, which included the between-subjects factors of Abstinence Day and M+B dose**,** showed a main effect of the Abstinence Day ($F_{1,22}$=27.2, p<0.001), M+B dose ($F_{1,22}$=19.7, p<0.001) and interaction of Abstinence Day X M+B dose ($F_{1,22}$=13.6, p=0.001). In contrast, shell injections of M+B had no effect on methamphetamine seeking in the extinction tests at abstinence day 15 and abstinence day 1 (Fig. 2F).

We also analyzed the time course of extinction responding on abstinence day 15 using the between-subject factors NAc Subregion and M+B dose, and the within-subject factor Session Time (20, 40 and 60 minutes; Fig. 2G). The statistical analysis showed a main effect of M+B dose ($F_{1,27}$=16.2, p<0.001), Session Time ($F_{2,27}$=28.6, p<0.001), NAc Subregion ($F_{1,27}$=9.3, p=0.006) and interaction between the M+B dose and NAc Subregion ($F_{1,27}$=6.4, p=0.018).

Finally, to rule out that the NAc core effect was due to a motor deficit, we trained all the NAc core abstinence day 15 group to self-administer SM1% solution (Fig. S2B). We than determined the effect of vehicle or M+B injections into the NAc core on ongoing SM1% solution-reinforced responding: we found that M+B injections into NAc core had no effect on SM1% solution self-administration ($F_{1,9}$=0.2, p=0.644) (Fig. S2C).

Exp. 3: Effect of NAc core SCH39166 or raclopride injections on incubation of methamphetamine craving

The timeline of the experiment is shown in Fig. 3A.

SM1% solution and methamphetamine training: The rats increased the rewards intake over sessions (Fig. S1B, left panel). The statistical analysis showed a significant effect of session for SM1% solution ($F_{5,100}$=31.9, p<0.001) and methamphetamine ($F_{11,220}$=25.0, p<0.001). During the three discrete choice sessions, the rats showed a strong preference for the SM1% solution ($F_{1,20}$=40.8, p<0.001) (Fig. S1B, right panel).

Voluntary abstinence: During the 14 days of food choice-induced abstinence the rats showed a strong preference for the SM1% solution (Fig. 3B). The statistical analysis showed a significant effect of reward type ($F_{1,20}$=2611.2, p<0.001).

Relapse test: NAc core injections of raclopride or SCH 39166 decreased methamphetamine seeking in the extinction tests at abstinence day 15 ($F_{2,17}$=4.3, p<0.05; main effect of dopamine antagonist condition) (Fig. 3C). We also analyzed the time course of extinction responding using the between-subjects factor of dopamine antagonist condition and the within-subjects factor of Session (20, 40 and 60 minutes; Fig. 3D). The statistical analysis showed a main effect of the dopamine antagonist condition ($F_{2,18}$=5.5, p<0.05), Session Time ($F_{2,36}$=31.3, p<0.001) and an interaction between the dopamine antagonist condition and Session time ($F_{4,36}$=6.6, p<0.001).

Finally, to rule out that the effect was due to a motor deficit, we trained all the rats to self-administer SM1% solution (Fig. S2D) and determined the effect of injections into the NAc core on ongoing SM1% solution-reinforced responding. We found that raclopride or SCH 39166 injections into NAc core had no effect on SM1% solution self-administration ($F_{2,17}$=0.2, p=0.8) (Fig. S2E).

Exp. 4: Effect of NAc core flupenthixol injections on incubation of methamphetamine craving

The timeline of the experiment is shown in Fig. 3A.

SM1% solution and methamphetamine training: The rats increased the rewards intake over sessions (Fig. S1C, left panel). The statistical analysis showed a significant effect of session for SM1% solution ($F_{5,65}$=2.620, p<0.05) and methamphetamine ($F_{11,143}$=3.422, p<0.05). During the three discrete choice sessions, the rats showed a strong preference for the SM1% solution ($F_{1,13}$=10.3, p<0.01) (Fig. S1C, right panel).

Voluntary abstinence: During the 14 days of food choice-induced abstinence, the rats showed a strong preference for the SM1% solution (Fig. 3E). The statistical analysis showed a significant effect of reward type ($F_{1,13}$=82.9, p<0.001).

Relapse test: NAc core injections of flupenthixol decreased methamphetamine seeking in the extinction test at abstinence day 15 ($F_{1,12}$=7.3, p= p<0.05; main effect of dopamine antagonist condition) (Fig. 3F). We also analyzed the time course of extinction responding using the between-subjects factor of dopamine antagonist condition and the within-subjects factor of Time (20, 40 and 60 minutes; Fig. 3G). The statistical analysis showed a main effect of Time ($F_{2,24}$=13.0, p<0.001).

The timeline of the dose effect curve of flupenthixol is shown in Fig. S3A.

SM1% solution training: The rats increased the rewards intake over sessions (Fig. S3B). The statistical analysis showed a significant effect of session for SM1% solution ($F_{4,24}$=9.0, p<0.01).

Dose effect curve: Flupenthixol dose dependently decreased SM1% solution reinforced responding (Fig. S3C). The statistical analysis showed a significant effect of Dose ($F_{4,24}$=8.7, p<0.01). A post hoc analysis conducted with the Least Significant-Difference test revealed a significant difference between saline and the doses of flupenthixol of 30 and 40 μg/side and a nearly significant effect at the dose of 20 μg/side.

Discussion

There are three main findings in our study. First, incubation of methamphetamine craving was associated with increased Fos expression in NAc core Drd1- and Drd2-MSNs after 15 abstinence days; in contrast we did not found any increased Fos expression in the NAc shell subregion. Second, reversible inactivation of NAc core selectively decreased methamphetamine seeking during late (day 15) but not early (day 1) abstinence. This effect was selective to methamphetamine seeking, muscimol + baclofen injections had no effect on SM1% solution-reinforced responding test. Third, blockade of Drd1-, Drd2- or both Drd1-Drd2-family receptors in NAc core decreased incubated (day 15) methamphetamine craving after voluntary abstinence to a similar extent. Together, our results indicate that dopamine transmission through Drd1 and Drd2 in activated NAc core is critical to the incubation of methamphetamine craving after food choice-induced voluntary abstinence.

Methodological considerations

A number of methodological issues should be considered in the interpretation of the present data. The first issue is represented by the specificity of the inactivating effect of muscimol + baclofen on NAc core. It is unlikely that this effect was due to non-specific performance deficits, as muscimol + baclofen had no effect on lever presses during abstinence day 1 or high-rate operant responding for the palatable solution (sucrose 1% + maltodextrin 1%) (see Results and Fig. 2E-S2C). Similarly, it is unlikely that the effect of SCH39166 and raclopride NAc core injections was due to non-specific performance deficits. This is because we used a dose that had no effect on operant responding for the palatable food (%) (see Results and Fig. S2E). In this context, it is important to emphasize that depletions of NAc dopamine do not substantially impair all aspects of primary food motivation and that NAc infusions of dopamine antagonists at doses that impair runway performance did not impair sucrose intake (for reviews [41,42]).

The second methodological issue is represented by the anatomical specificity of the intracranial injections. It is unlikely that the behavioral changes were due to drug diffusion from the injection site [43], because injections into NAc shell had no effect on incubated methamphetamine craving after food choice-induced voluntary abstinence (see Results and Fig. 2F). A third methodological consideration is that we used only male rats in this study. In this regard, several studies demonstrated sex differences in psychostimulant self-administration and relapse, including incubation of cocaine craving after forced abstinence [44,45], and reinstatement of methamphetamine seeking [46]. However, it is unlikely that female rats would have responded differently to our manipulations. In our previous studies, we found no evidence for sex differences in methamphetamine self-administration, or in the strong preference for the palatable food over methamphetamine or the magnitude of incubated methamphetamine seeking after either forced or voluntary abstinence [11]. The fourth methodological issue concerns the muscimol+baclofen effect on methamphetamine seeking. We only observed an effect of the two agonists on abstinence day 15 but not day 1, suggesting a selective effect on ‘incubated’ cue-induced drug seeking. However, this selective time-dependent effect should be interpreted with caution because of a potential floor effect due to low responding on day 1.

Finally, from a brain mechanism perspective, we did not investigate the role of dopamine transmission in the NAc shell in the incubation of methamphetamine craving after voluntary

abstinence because we did not observe neither a time-dependent increase in Fos expression after the relapse tests on abstinence days 1 and 15 (see Results and Fig. 1G, lower panel) nor an attenuation of incubated craving after the reversible inactivation with muscimol + baclofen (see Results and Fig. 2F). However, these correlational and causal data do not definitively rule out a role of NAc shell dopamine transmission in the incubation of methamphetamine craving because previous studies have demonstrated dissociable effects of reversible inactivation relative to dopamine or glutamate receptors blockade [47].

The role of nucleus accumbens core in incubation of methamphetamine craving after voluntary abstinence

The NAc core and shell are heterogeneous structures distinguished on the basis of the morphology and projections [48,49]. These neuroanatomical evidences stimulated a large number of studies on the role of NAc core and shell in motivated behavior [50,51] and conditioned and unconditioned rewarding effects of drugs [52,53]. Our results from Exp. 1 and 2, supporting a role for the NAc core but not shell in incubation of methamphetamine craving after voluntary abstinence, are in agreement with the general notion that NAc core and shell mediate distinct aspects of drug-motivated behaviors [54]. In particular, they are in agreement with previous findings where permanent lesions or reversible inactivation (muscimol + baclofen) of core but not shell decrease discrete-cue-induced reinstatement of cocaine [55,56] and methamphetamine seeking [57] and discrete-cue-induced cocaine seeking, as assessed in an acquisition of a new response procedure [58].

Our results provide further correlational evidences of altered firing in the NAc core during incubation of cocaine craving after forced abstinence [22,23]. This altered firing is putatively associated with the higher conductance expressed by the CP-AMPARs (homomeric GluA1 receptors) that accumulate in the NAc core over time [24,59]. Consistent with these findings, incubated cocaine and methamphetamine craving after forced abstinence was reduced by decreasing CP-AMPARs transmission in NAc core [24-26] or, for cocaine, via weakening the prelimbic cortex to NAc core pathway [27].

Levels of CP-AMPARs are also elevated in the NAc shell after incubation of cocaine craving [27,29,31] and CP-AMPARs transmission in the NAc shell play a causal role in incubation of cocaine

craving. These latter evidences are at odds with our data. In Exp. 1 (RNAscope in situ hybridization for Fos) and Exp. 2 (muscimol + baclofen inactivation) we did not observe any appreciable increase in Fos expression (marker of neuronal activity) nor an appreciable reduction in incubated methamphetamine craving after voluntary abstinence. What may account for this discrepancy? The answer is not straightforward because a direct comparison of our study and Dong and colleagues' studies [27,31] is not possible for two main reasons: 1) we used methamphetamine while Dong and colleagues used cocaine. In the case of NAc core, both incubation to cocaine and methamphetamine have been shown to result in CP-AMPARs in the core. However, we do not know if incubation to methamphetamine results in CP-AMPARs in the shell; 2) we used an extended drug-self administration procedure for 12 days (6 h/day) and voluntary abstinence in adult rats, while Dong and colleagues used a single overnight training session plus short-access procedure for 5 days (2 h/day) and forced abstinence in juvenile rats. It is well established that the neural adaptions occurring during an extended-access relative to short-access self-administration are distinct [60] and that juvenile onset and adult onset self-administration are associated with different physiological and behavioral changes [61,62].

Finally, the neuroanatomical substrates responsible for the expression of incubated methamphetamine craving, identified in this (NAc core but not shell) and previous studies (CeA but not BLA; [20,63]), mirror the neuroanatomical substrates involved in the expression of the 'general' form of Pavlovian to Instrumental Transfer (PIT) [64], which is thought to represent the general motivational or affective properties of the reward [65]. First proposed by Li and colleagues [63], our findings support the hypothesis that incubation of drug craving may be due to time-dependent increases in the motivational potency of Pavlovian drug associated cues after abstinence that is mediated by increased activity of CeA and NAc core during the late abstinence relapse tests. The common role of CeA [20,63] and NAc core in promoting incubation of methamphetamine craving suggests that these regions, with no direct anatomical connections [66] interact functionally during the drug seeking test. As previously speculated for PIT [64], the CeA may indirectly recruit the NAc core via its projections to ventral tegmental area, which projects to NAc core [66].

Role of NAc Drd1- and Drd2-MSNs during incubation of methamphetamine-craving after voluntary abstinence

The mesolimbic dopamine system innervating the NAc is critically involved in cue-elicited reinstatement of drug-seeking [67]. However, relatively little is known on the role of NAc-dopamine transmission in incubation of cue-induced drug craving after prolonged forced and voluntary abstinence. Ito et al., [68] first reported that cocaine-associated cues significantly increase extracellular dopamine in the NAc core (see Willuhn for a review and conflicting findings [69]), suggesting that enhanced dopamine transmission may be involved in cue-induced craving and relapse to drug seeking. Subsequently, a seminal study by Ciccocioppo et al. [70], showed that systemic blockade of Drd1 decreases both discriminative cue-induced reinstatement and discriminative cue-induced Fos and that the reinstatement effect of the discriminative cues persists for at least 4 months after cocaine exposure [70]. These investigators also demonstrated that the response to the cocaine-associated discriminative cues is remarkably persistent over repeated testing and that Drd2 play a role in this form of reinstatement [71]. Since then, several studies employing Drd1 and Drd2 partial agonists and antagonists have been shown to reduce cue-induced drug seeking (reviewed in [72]).

Overall, the effect of selective Drd1 and Drd2 (SCH39166 and raclopride) or both Drd1-Drd2 (flupenthixol) antagonists reported here confirm earlier findings and suggest that the incubation of methamphetamine-seeking after voluntary abstinence is not dependent of a specific subtype of MSNs. Furthermore, our data do not support the hypothesis that Drd1 and Drd2 MSNs act in opposing manner [73] but rather, suggests that a coincident and concerted MSNs activity is required for reward-related behaviors [74-76]. Indeed, regardless of the antagonist used in our experiments (SCH39166, Drd1-selective; raclopride, Drd2-selective; flupenthixol, Drd1-Drd2-selective), we observed reliable reduction of incubation of methamphetamine craving.

Our data are also consistent with our previous results on incubation of methamphetamine seeking after forced and voluntary abstinence. Indeed, in these studies the incubation-sensitive Fos-positive neurons (activated after periods of forced or voluntary abstinence) in the dorsomedial and dorsolateral striatum co-expressed both Drd1 and Drd2 [10,77]. Furthermore, studies employing extended-access

cocaine regimens leading to incubation of cocaine craving support the idea that there is a similar plasticity in Drd1- and Drd2-expressing MSNs in NAc core [78] or even in the dorsal striatum [79]. An important example is the elevation of CP-AMPAR levels that is observed in nearly all NAc core MSNs after extended-access cocaine self-administration and after a month of abstinence, albeit in a pathway-specific manner [59,78].

Conclusions

We used our recently developed choice-based rat model of drug relapse after voluntary abstinence that mimics the human condition of relapse after successful contingency management. Here we used RNAscope in situ hybridization and pharmacological approaches to show a critical role of Drd-1 and Drd2-mediated NAc core neuronal activity in the expression of incubation of methamphetamine craving after food choice-induced voluntary abstinence.

To the degree that our choice-based model of drug relapse in rats mimics the human condition of relapse after successful contingency management, our findings suggest the NAc core as a potential target for relapse prevention.

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Figure Legends

Figure 1. *Incubation of methamphetamine craving is associated with activation of NAc core but not shell: behavioral data and RNAscope data.* (**A**) Timeline of the experiment (Exp.1). The self-administration chamber is equipped with two active levers (SM1% and methamphetamine), one inactive lever, one discriminative stimulus (yellow house light), two conditioned stimuli (white light for methamphetamine, triple-light for SM1%), two pumps (one for SM1%, one for methamphetamine), a liquid receptacle (**B**) Self-Administration training. Mean ± SEM number of SM1% rewards (0.4 ml/reward) or methamphetamine infusions (0.1 mg/kg/infusion; 0.1 ml/infusion) during the 6-hour sessions, n=16. (**C**) Choice sessions during training. Mean ± SEM number of SM1% rewards and methamphetamine infusions earned during the three discrete choice sessions during training (20 trials every 10 minutes), n=16. (**D**) Voluntary abstinence. Mean ± SEM number of SM1% rewards and methamphetamine infusions earned during 14 discrete choice sessions, n=10. (**E**) Relapse tests. Mean ± SEM number of lever presses on active, non-reinforced, and inactive levers during the 60-minute test sessions. (**F**) Representative photomicrographs of the NAc core and NAc shell and Fos and Drd1 or Drd2 labeling in the relapse-test and no-test groups. (Fos, white; Drd1, green; Drd2, red; DAPI, blue). Arrows indicate representative cells. (**G**) Fos neurons quantification. Number of Fos-IR (immunoreactivity) nuclei per mm^2 in NAc core (top panel) and shell (bottom panel). (**H**) Fos-IR co-expression with Drd1 cells in NAc core (top panel) and shell (bottom panel). (**I**) Fos-IR co-expression with Drd2 cells in NAc core (top panel) and shell (bottom panel). * $p<0.05$ different from day 1. Circles represent individual data, n=16, 3-5/group.

Figure 2. *Reversible inactivation of NAc core but not shell decreased incubated methamphetamine seeking after voluntary abstinence.* (**A**) Timeline of the experiment (Exp.2). (**B**) Self-administration training. Mean ± SEM number of SM1% rewards or methamphetamine infusions during the 6-hour sessions in rats with implanted cannula in the NAc core (Core) or NAc shell (Shell), n=52. (**C**) Choice sessions during training. Mean ± SEM number of SM1% rewards and methamphetamine infusions earned during the three discrete choice sessions during training in Core and Shell rats, n=52. (**D**) Voluntary abstinence. Mean ± SEM number of SM1% rewards and methamphetamine infusions earned during 14 discrete choice sessions from the voluntary abstinence

group tested on abstinence day 15, n=28. (**E-F**) Relapse tests for muscimol + baclofen (M+B) injections into NAc core and shell. Mean ± SEM number of lever presses on active, non-reinforced lever during the 60-minute test sessions on day 1 (n=24) or day 15 (n=28) of voluntary abstinence. We injected vehicle or M+B (50 ng+50 ng/0.5 μl/side) into NAc core or shell 15 minutes before the 60-minute extinction tests. We added a representative photomicrograph of the cannula placement in the area (scale bar, 500 μm). * $p<0.05$ different from day 1. (**G**) Time course of lever presses during the relapse test on day 15. Mean ± SEM number of lever presses on active, non-reinforced lever during different time periods of the relapse test (0-20, 20-40, 40-60 min) (n=28). (**H-I**) Cannula placement in NAc core or shell. Approximate placement (mm from bregma) of the injector tips [80]. Vehicle: open circles; M+B: closed circles).

Figure 3. *Drd1***,** *Drd2 or Drd1-Drd2 antagonists' injection into NAc core decreased relapse after voluntary abstinence.* (**A**) Timeline of the experiments (Exp.3 and 4). (**B**) Voluntary abstinence Exp.3 (raclopride or SCH 39166). Mean ± SEM number of SM1% rewards and methamphetamine infusions earned during 14 discrete choice sessions. (**C**) Relapse test for raclopride or SCH 39166 injections into NAc core (Exp.3). Mean ± SEM number of lever presses on active, non-reinforced, and inactive levers during the 60-minute test sessions on day 15 of voluntary abstinence. We injected vehicle or raclopride (1.0 μg/0.5 μl/side) or SCH 39166 (1.0 μg/0.5 μl/side) into NAc core 15 minutes before the 60-minute extinction tests. We added a representative photomicrograph of the cannula placement in the area (scale bar, 500 mm). (**D**) Time course of lever presses during the relapse test on day 15 (Exp.3). Mean ± SEM number of lever presses on active, non-reinforced lever during different time periods of the relapse test (0-20, 20-40, 40-60 min). (**E**) Voluntary abstinence Exp.4 (flupenthixol). Mean ± SEM number of SM1% rewards and methamphetamine infusions earned during 14 discrete choice sessions. (**F**) Relapse test for flupenthixol injections into NAc core (Exp.4). Mean ± SEM number of lever presses on active, non-reinforced, and inactive levers during the 60-minute test sessions on day 15 of voluntary abstinence. We injected vehicle or flupenthixol (10 μg/0.5 μl/side) into NAc core 15 minutes before the 60-minute extinction test. We added a representative photomicrograph of the cannula placement in the area (scale bar, 500 mm). (**G**) Time course of lever presses during the relapse test on day 15 (Exp.4). Mean ± SEM number of lever presses on active,

non-reinforced lever during different time periods of the relapse test (0-20, 20-40, 40-60-minute). (**H**) Cannula placement in NAc core (Exp.3). Approximate placement (mm from bregma) of the injector tips [80]. Vehicle: open circles; raclopride: closed squares; SCH 39166: open triangles). (**I**) Cannula placement in NAc core (Exp.4). Approximate placement (mm from bregma) of the injector tips [80]. Vehicle: open circles; flupenthixol: closed circles). Exp.3 (n=21, 6-8/group); Exp.4 (n=14, 7/group). * $p<0.05$ different from saline.